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A DETERMINATION OF THE CHARACTER OF ACIDS PRODUCED BY HEMOLYTIC AND NON-HEMOLYTIC STREPTOCOCCI FROM PATHOGENIC SOURCES AND FROM MILK.

by

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INTRODUCTION

The search for characters which will differentiate pathogenic from non-pathogenic streptococci has occupied the minds of many bacteriologists for nearly twenty years. Since the work of Gordon (1904), many efforts have been made to differentiate streptococci on the basis of the fermentation of carbohydrates.

Studies of fermentation reactions by the streptococcus group have been approached from several angles. Early workers, headed by Gordon, sought differentiation by the ability or inability of the organisms to ferment certain substances. Later, the amount of acid produced, as determined by titration, was considered of importance. More recently the work of Clark and Lubs on the differentiation of members of the colon group by determination of the limiting hydrogen-ion concentration has lead to the application of this phase of fermentation study to the streptococci.

So far, little attention has been paid to the products of fermentation or to the kinds of acids produced. Evans (1914 and 1918) has studied the substances formed by five strains of streptococci cermed with cheese ripening, and Foster (1921) has thoroughly investigated the fermentative and putrefactive products of one strain of Streptococcus hemolyticus from a fatal case of bronchopneumonia.

As these previous biochemical studies were concerned with streptococci from but two sources, there is an open field for more extended research.

The purpose of this investigation is to determine the character and ratio of the acids produced by hemolytic and non-hemolytic streptococci with the hope of discerning distinctive differential features in their acid products.

The study included first, an identification of the organisms, second, a comparison of titratable acidity with limiting hydrogen-ion concentration, third, a determination of the proportion of volatile and non-volatile acids with an investigation of the amounts of each produced daily, and fourth, a qualitative, quantitative, and graphical study of the kind of acids formed.

II. REVIEW OF LITERATURE ON FERMENTATION REACTIONS OF STREPTOCOCCI.

Attempts to differentiate streptococci were unsuccessful until Gordon (1904), in looking for additional tests for bacterial sewage contamination of water, tested the fermentation reactions on various carbohydrates. After testing many sugars with a great number of strains of streptococci from sewage, he concluded that they could be grouped more or less definitely by their action on nine

carbohydrates. These have since been called "Gordon's tests". Following Gordon's work, Andrews and Horder (1906), Winslow (1912), Hopkins and Lang (1914), Kligler (1915), and many others have thoroughly studied fermentation reactions and grouped streptococci according to their ability to ferment the various carbohydrates. Lyall (1914), Holman (1916), Blake (1917), and Dible (1921) have described classifications based on hemolytic and fermentative reactions. Broadhurst (1915) found that the fermentation reactions aside from differences due to rate of growth, are remarkably constant under various environmental conditions. Salter (1921) claimed a greater range of fermenting power for the pathogenic hemolytic streptococci than for the milk strains.

Heineman (1906) stated that Strep. lacticus agreed in morphological and cultural properties with pathogenic, fecal, and sewage streptococci. Walker (1910), Bergey (1912), Buerger (1907), Thro (1914-15) and Beattie(1921) questioned the value of fermentation tests in carbohydrates because of their variable reactions. Kinsella (1918) found that the older classification of streptococci based on morphology and variations of cultural characteristics had proved unsatisfactory because the criteria did not bear any relation to the more fundamental questions of distribution in man, and pathogenicity.

Broadhurst (1912-13), Stowell, Hilliard, and Schlesinger(1913), Smith and Brown (1914-15) showed that there was a difference in acidity produced on dextrose bouillon between strains of hemolytic streptococci from human and bovine sources - the acidity being determined by titration. Maximum acidity produced in the culture medium by streptococci of bovine type was the basis of differentiation used by Avery and Cullen (1919) to distinguish between streptococci of human and bovine origin. Hopkins and Lang (1914) stated that titration of the amount of acid formed in fermentation were of no value in classification, while Jones F. (1920) believed that titration was as satisfactory as hydrogen-ion values.

Ayers, Johnson, and Davis (1918) found in dextrose-yeast-peptone medium, that pathogenic streptococci reached a limiting hydrogen-ion concentration from 5.4 to 6.0, while a non-pathogenic strain reached a higher concentration of pH 4.5 to 4.9. Brown J.H. (1920) showed that the pH limit for bovine strains was 4.5 to 4.6, while that for human was 5.1 to 5.4. Jones H. (1920) and Arnold L. (1921) recorded a difference between the limiting hydrogen-ion concentration for streptococci grown on one percent sugar bouillons. Jones concluded that, "the amount of glucose which a given organism can consume is influenced by

buffer content of the medium, that is, by such constituents as phosphates, proteins, etc., which aid in holding the concentration of hydrogen-ions from toxic limit, permitting a large amount of sugar to be decomposed."

A general view of the literature indicates that the majority of observers have found the fermentation tests constant enough to warrant a division of the streptococcus group into a number of distinct varieties and at least into those of bovine and human origin.

III. METHODS OF PROCEDURE.

In the attempt to determine the character of the acids produced by hemolytic and non-hemolytic streptococci, it was necessary to have the cultures produce a sufficient quantity of acid for experimental purposes. Flasks containing 100 cc. of one percent infusion broth were inoculated with pure strains of streptococci and allowed to grow until fermentation was complete. Each day the amount of acid formed was determined by titration and the entire contents of the flasks were neutralized. At the end of the fermentation period the volatile acids were separated from the non-volatile by steam distillation. Qualitative, quantitative, and graphical tests were then applied to determine the character of the individual acids constituting

these larger groups.

a. Sources of Cultures.

Cultures from pathogenic conditions were obtained from the bacteriological laboratory of the Johns Hopkins Hospital during the months of June and July 1922, from the Hygienic Laboratory in Washington, from the laboratory of H.K.Mulford Company, and from stock cultures at the bacteriological laboratory of the Johns Hopkins School of Hygiene. See Table I.

The streptococci from milk were obtained by plating samples of unpasteurized milk secured from one of the dairies in Baltimore during the month of July 1922.

b. Media employed.

Pure strains of streptococci were transferred to Huntoon's infusion broth, incubated eighteen hours, then plated on blood agar (0.5 cc. citrated rabbit's blood, 10 cc. infusion agar). Hemolysis was noted here, but again confirmed in infusion broth tubes which had been incubated eighteen hours, then had 0.2 cc. of a 5 percent solution of rabbit or sheep cells added and incubated for one hour longer.

Colonies were fished from plates to blood agar slants (0.2 cc. rabbit's blood, 5 cc. infusion agar), and incubated 24 hours. Tubes of Huntoon's plain broth were inoculated from the

Table I.

Sources of Cultures of Hemolytic Streptococci.

Culture Number	Obtained	Source
389	Hygienic Laboratory	Unknown
388	" "	"
390	" "	"
1459	Mulford's Laboratory	"
H-131	Baltimore Dairy	Pasteurized milk
H-23	" "	" "
133 a	School of Hygiene	Throat
133 b	" " "	"
228 a	" " "	Endocarditis
228 R	" " "	"
1681	Hopkins Hospital	Antrum
1613	" "	"
164	" "	"
1605	" "	"
163	" "	"
165	" "	"
171	" "	"
166	" "	"
161	" "	"
169	" "	"
1213	" "	Endocarditis
136	" "	Nose
46	" "	"
237 (1)	" "	Erysipelas
237 (2)	" "	"
35	" "	Deep wound
W-3	" "	Throat
26 R	" "	Mastoid-right
26 L	" "	Mastoid-left
84	" "	Tonsil
262	" "	Empyema
215	" "	"

blood agar slants, and incubated 18 to 24 hours. The initial pH of all the broth used was 7.2 - 7.4, Later, in the work, infusion broth was substituted entirely for Huntoon's unfiltered broth, but no marked differences were noted in the final results obtained. One percent lactose, saccharose, and dextrose sugars were used throughout all the experiments.

One half cc. of a culture in Huntoon's plain broth was used to inoculate flasks containing 100cc. Huntoon's broth plus one percent of sugar. The sterile sugars were added after the flasks containing the broth had been autoclaved, in order that no caramelization of the sugar should take place. The broth used in each experiment was made from the same meat and titrated in the same container, so that its salt content and hydrogen-ion concentration was uniform.

The flasks were provided with sterile rubber stoppers to prevent evaporation, and were uniform in size and shape, so that the surface exposed was the same in all. The flasks were always returned to their same positions in the incubator, incubated at 37° C., and examined each day at the same hour, so that as nearly as possible the conditions would be alike.

c. Titration of Broth Cultures in Flasks.

1. Method of Direct Titration.

Procedure for Examination.

1. Five tenths of a cc. of culture was removed from each flask with a sterile pipette, and added to small tubes containing 2 cc. sterile distilled water, and 0.2 cc. phenolsulphonephthalein indicator.

2. To 0.5 cc. of the culture, N/20 KOH was added drop by drop until the pH value, as indicated by comparison with standard buffer solutions, was the same as that of the original broth.

Computation.

1. If on the first day of titration, it required 0.35 cc. of N/20 KOH to bring 0.5 cc. of the culture back to the original pH value of the broth before inoculation, and 99.5 cc. of culture remained in the flask, then -

$$99.5 \times 0.35 \times 2 = (34.825 \times 2) \text{ cc.N/20 KOH.}$$

In order to add less volume, but obtain the same result, N/1 KOH was added. Therefore,

$$34.825 \times 2 \div 20 = 3.48 \text{ cc. N/1 KOH.}$$

2. Titrations were performed each day. The amount of N/1 KOH to be added was then calculated, always taking into consideration the 0.5 cc. of the culture withdrawn for the tests each day. This procedure was followed until no more acid was produced by the streptococci. The streptococci usually were living at this point, as was shown by plating a sample of the broth, but were unable to

produce further acid as the sugar was completely used up. See Table II for calculation.

2. Method of Titration by means of pH Values.

Foster (1921), in working with cultures of Strep. hemolyticus, established the fact that with increased concentrations of buffer salts there was an increased neutralizing power which delayed the production of the final acidity level, thus allowing the organism more time for fermentation.

Therefore it was necessary to discover the extent of the neutralizing power in each lot of broth used in the experiments. The percentage of sugar throughout the experiments was constant, and all broth used had the initial pH of 7.2. The only varying factor was the protein and salt content of the various batches of infusion broth.

In order to investigate the buffer effect of the broth, titration curves of broth and pure acetic acid were plotted. Owing to the fact that the lower volatile fatty acids do not vary greatly in their extent of ionization, acetic acid was used to make the experimental curves. The data were obtained in the following manner: To 1 cc. portions of one percent sugar infusion broth of pH 7.2, 1cc. amounts of increasing dilutions of acetic acid varying from 1 to 500 to 1 to 1000 were added, and the pH value determined. The pH values of the pure acetic acid dilutions were also recorded. The curves

Table II.

Computing Table

cc. of broth in flask	.55	.50	.45	.40	.35	.30	.25	.20	.15	.10	.05	cc.N/20 KO
												cc.N/1
99.5	5.46	4.97	4.47	3.98	3.48	2.98	2.48	1.99	1.49	.99	.49	
99.0	5.44	4.95	4.45	3.96	3.46	2.97	2.47	1.98	1.48	.99	.49	
98.5	5.41	4.92	4.43	3.94	3.44	2.95	2.46	1.97	1.47	.98	.49	
98.0	5.39	4.90	4.41	3.92	3.43	2.94	2.45	1.96	1.47	.98	.49	
97.5	5.35	4.87	4.38	3.90	3.41	2.92	2.43	1.95	1.46	.97	.48	
97.0	5.33	4.85	4.36	3.88	3.39	2.91	2.42	1.94	1.45	.97	.48	
96.5	5.30	4.82	4.34	3.86	3.37	2.89	2.41	1.93	1.44	.96	.48	
96.0	5.28	4.80	4.32	3.84	3.36	2.88	2.40	1.92	1.44	.96	.48	
95.5	5.24	4.77	4.29	3.82	3.34	2.86	2.39	1.91	1.43	.95	.47	
95.0	5.22	4.75	4.27	3.80	3.32	2.85	2.38	1.90	1.42	.95	.47	
94.5	5.19	4.72	4.25	3.78	3.30	2.82	2.37	1.89	1.41	.94	.47	
94.0						2.81	2.35	1.88	1.41	.94	.47	
93.5						2.80	2.33	1.87	1.40	.93	.46	
93.0						2.78	2.32	1.86	1.39	.93	.46	
92.5						2.76	2.31	1.85	1.38	.92	.46	
92.0						2.76	2.30	1.84	1.38	.92	.46	
91.5						2.74	2.28	1.83	1.37	.91	.45	
91.0						2.72	2.27	1.82	1.36	.91	.45	
90.5						2.70	2.26	1.81	1.35	.90	.45	
90.0						2.70	2.25	1.80	1.35	.90	.45	

The left hand column of figures represents cc. of culture remaining in the flask. The top row of figures represent cc. N/20 KOH required to bring back 0.5 cc. of the culture to the desired pH by titration. The remaining figures stand for the cc. N/1 KOH required to be added to the entire flask to bring it to the desired pH.

were plotted using pH values as abscissae and dilutions of acetic acid as ordinates. Reference to the curves (Graph I) will show that the infusion broth exerted a slight but distinct buffer effect. Two more graphs were plotted, the first representing titration of dilutions of acetic acid with N/20 KOH, using pH 7.2 as the end point. The numbers of cubic centimeters of alkali were used as abscissae, the dilutions of acid as ordinates. The second graph was made by titrating with N/20 KOH the known values of pH in acetic acid and broth plus acetic acid. The pH values were used as ordinates and the cubic centimeters of alkali as abscissae. See Graphs II and III.

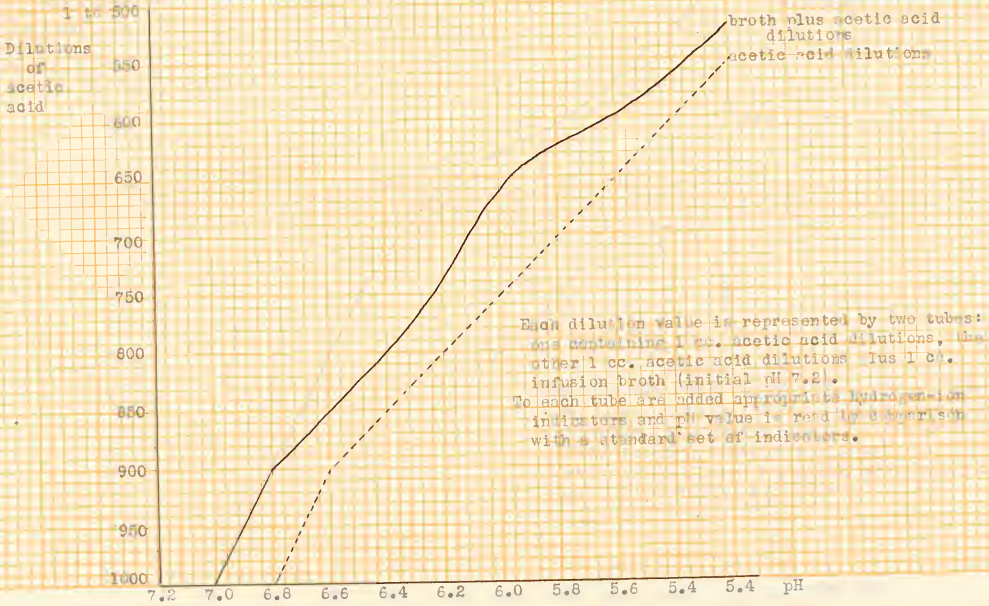
Graph III was the buffer curve desired. It was necessary to compute a new curve for each portion of broth made because of the variation in salts and protein. Slight deviations occurred but the curves were typically alike, and therefore only one set of graphs are shown.

In order to use the buffer curve in titration, suitable hydrogen-ion indicators were added to 0.5 cc. of broth sample removed from a flask, comarison was made with a standard set of hydrogen-ion tubes, the pH was determined, then from the curve was read the number of cc. N/20 KOH necessary to neutralize the acid formed in the broth. Reference was made to the Table I for computation,

Graph I.

Showing buffer effect of infusion broth.

1. The reaction of broth to changes in pH value by the addition of acetic acid is here indicated, and compared with that of the pure acid.



Each dilution value is represented by two tubes: one containing 1 cc. acetic acid dilutions, the other 1 cc. acetic acid dilutions plus 1 cc. infusion broth (initial pH 7.2).
To each tube are added appropriate hydrogen-ion indicators and pH value is read by comparison with a standard set of indicators.

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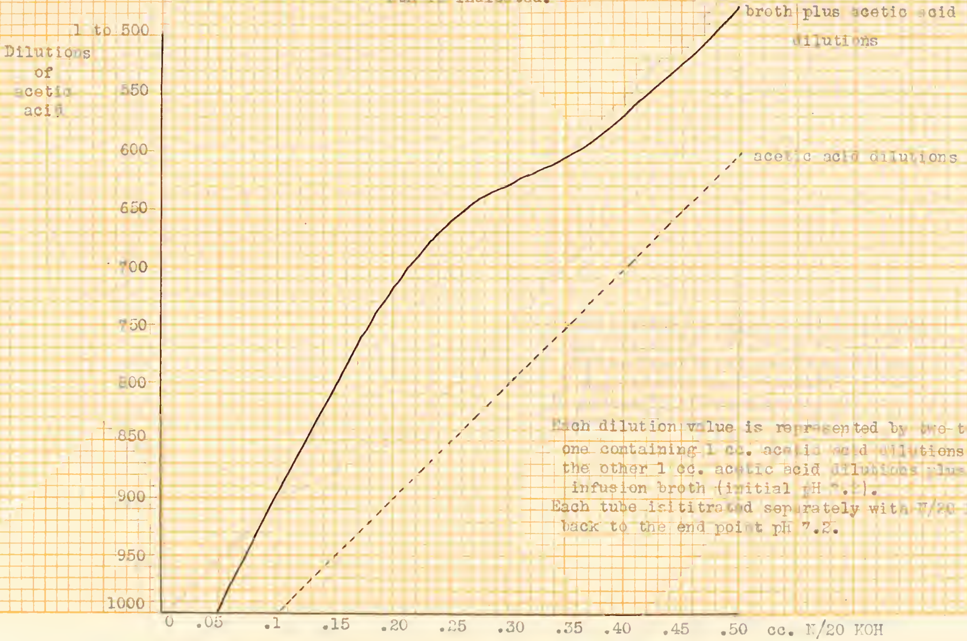
Graph II.
Showing buffer effect of infusion broth
II. The resistance of broth to the change in titratable acidity, by the addition of KOH is indicated.



Each dilution value is represented by two tubes: one containing 1 cc. acetic acid dilutions, and the other 1 cc. acetic acid dilutions plus 1 cc. infusion broth (initial pH 7.2).
Each tube is titrated separately with N/20 KOH back to the end point pH 7.2.

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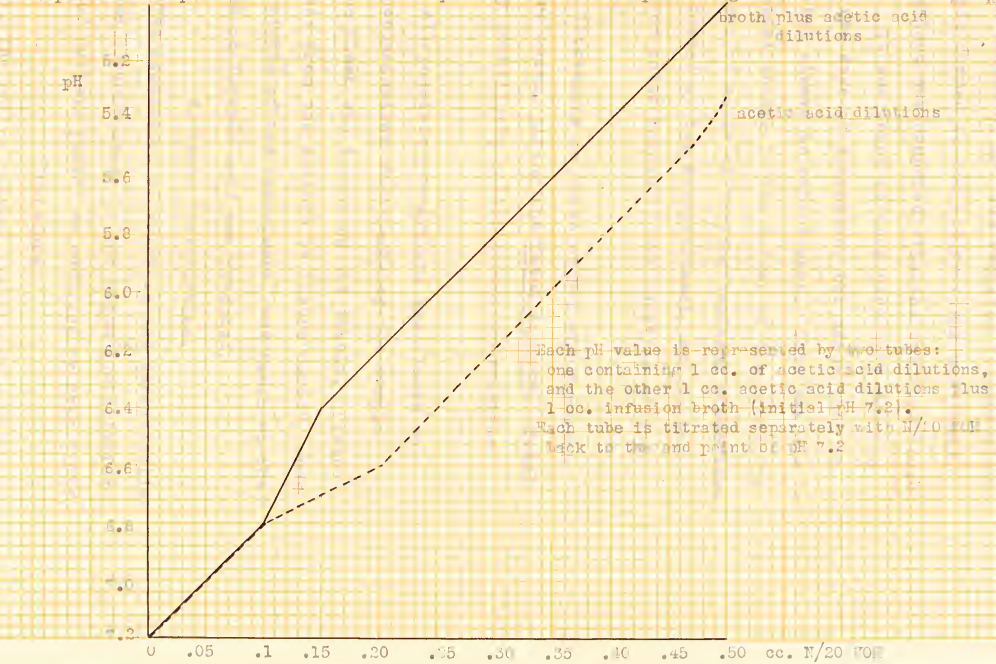
Graph III.

Showing buffer effect of infusion broth.

III. pH values of pure acetic acid and broth plus acetic acid are plotted against titration with N/20 KOH



Each pH value is represented by two tubes: one containing 1 cc. of acetic acid dilutions, and the other 1 cc. acetic acid dilutions plus 1 cc. infusion broth (initial pH 7.2). Each tube is titrated separately with N/10 KOH back to the end point of pH 7.2

and the amount of N/1 KOH required to neutralize the acid formed in the entire flask was found. This method was compared with the one first outlined. It was found to work very well for the first few days of titration, but after that it could not always be relied upon. This was due to a physical change in the buffer of the broth, to a mixture of acids of varying ionizing properties being formed by the strepøtococci, and to the potassium salts formed by the addition of the alkali.

Titrations in value of N/1 KOH for each day were added. giving the Total Acidity. Total acidity constitutes volatile and non-volatile acids.

All possible precautions were taken to avoid contamination when removing the sample from the flask and in adding the alkali. Plates were made from the culture to insure purity, once in the middle of the experiment, and again at the end when no further acid was produced.

d. Steam Distillation.

The separation of volatile and non-volatile acids was brought about by the steam distillation method of Duclaux (1900) as modified by Dyer (1916)

Procedure.

1. The entire culture was transferred to a large distilling flask, and 200 cc. of distilled

water added.

2) Ten drops of concentrated sulphuric acid were added to liberate the volatile acids from their potassium salts, and a small piece of paraffin added to lessen the foaming.

3) The apparatus was set up as illustrated in Plate I.

4) The distillation was carried on until 500 cc. of distillate had passed over.

1. Titration of Distillate.

1) The entire distillate was titrated with N/20 KOH using phenolsulphonephthalein as the indicator. The result was then converted into terms of N/1 KOH.

2) Test for possible sulphates which might have been carried over into the distillate was made with barium hydroxide solution, but none were found.

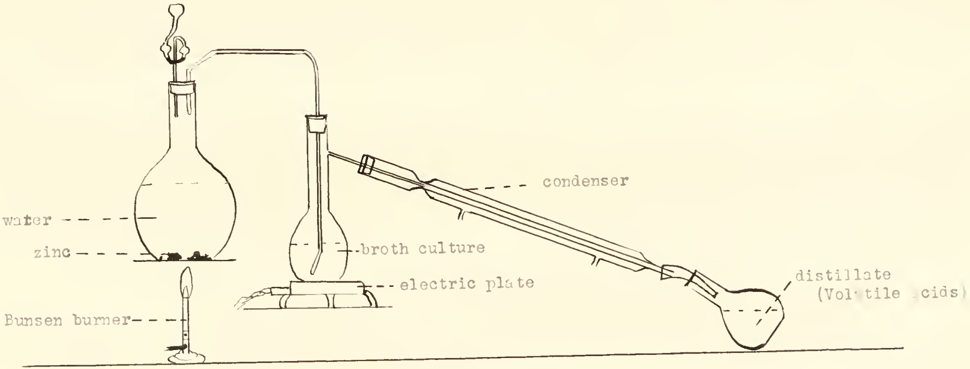
3) The titration of the distillate indicated the volatile acids present. This result was subtracted from the value of the total acidity, and divided into the remainder to give the ratio of volatile to non-volatile acids, as :

Volatile acids = 1.27 cc. N/1 KOH
Total acidity = 10.20 cc. N/1 KOH
Non-volatile = 10.20 - 1.27 = 8.93 cc.

8.93 ÷ 1.27 = 7 , Therefore, calling the amount of volatile acid "one", the ratio of volatile to non-volatile is as one is to seven.

Plate I.

Apparatus used in the Determination of Volatile Acids.



IV. DISCUSSION OF THE ACIDITY OF THE BROTH CULTURES.

The first and second day titrations were characterized by nearly twice as much acid production by the non-hemolytic streptococci as by the hemolytic, regardless of which sugar broth was fermented. The final total acidity varied but little in each sugar, although the highest acidity in all but a very few cases was produced in dextrose broth. The growth of the hemolytic streptococci was maintained longer than the non-hemolytic; they fermented a smaller amount of sugar each day, but in the end the total acidity was about equal. Table III shows that characteristic daily production by representative hemolytic and non-hemolytic organisms. The table indicates that the acid production for Strep. hemolyticus (Holman) is continued for six days longer than that of Strep. fecalis (Holman), but the total acidity varies but slightly.

From the repeated experiments in titrating each day's fermentation, it was observed that the titratable acidity for each sample was comparable with the rise or fall of the pH of that sample. See Table III. The titration method was found to be more reliable and accurate for continuous routine work, and especially when the record was kept until the end of the acid production period, at which time the buffer content was apt to change. For the first

Table III.

Comparison of pH Values with Daily Acid Production by Hemolytic and Non-hemolytic Streptococci.

		Hemolytic 133 a Strep. hemolyticus ii (Holman)		Non-hemolytic J-5 Strep. fecalis (Holman)	
Days	1	pH 6.2	1.96 cc.normal acid	pH 5.6	3.48 cc. normal acid
"	2	pH 6.4	1.46	pH 5.8	2.97
"	3	pH 6.4	1.45	pH 6.2	1.97
"	4	pH 6.4	1.45	pH 6.4	1.47
"	5	pH 6.4	1.44	pH 6.6	.97
"	6	pH 6.6	.96	pH 6.6	.97
"	7	pH 6.6	.95	pH 7.0	.48
"	8	pH 6.6	.95	pH 7.2	-
"	9	pH 7.0	.47	pH 7.2	-
"	10	pH 7.0	.47		12.31 cc.normal acid
"	11	pH 7.0	.46		
"	12	pH 7.1	.23		
"	13	pH 7.2	-		
"	14	pH 7.2	-		
"	15	pH 7.1	.22		
"	16	pH 7.2	-		
"	17	pH 7.2	-		
			12.45 cc.normal acid		

few days of growth, the determination of limiting hydrogen-ion values was equally as satisfactory as titration, so long as it was carried out in the same lot of broth and with the same pH as the end point for titration.

Tables IV and V are the records of acid production by hemolytic and non-hemolytic streptococci in dextrose, lactose, and saccharose broths, with the ratios of volatile to non-volatile acid content found in each. The numbers recorded for the ratios are derived from the averages of the volatile and non-volatile acid production found in four duplicate experiments performed at the same time, under like conditions with flasks inoculated with equal amounts of the same seed culture and grown in broth from one infusion. The blanks occurring in the lactose and saccharose broths are due, in nearly all cases, to the inability of the organisms to ferment that sugar.

Henrici (1916) stated that, " The carbohydrate fermentation tests are of no significance from the standpoint of virulence, and of slight or doubtful significance from the standpoint of tissue localization. The experiments performed supported this contention, for there was no constancy in the total acidity for the same strain of organisms; as much variation occurred with one sugar as another, neither could any significance be attached to the sources from which the hemolytic streptococci had come. Attempts to group the organ-

Table IV.

Acid Production by Hemolytic Streptococci in Dextrose, Lactose, and Saccharose Broths, with the Ratios of Volatile to Non-volatile Acid Content in Each.

Hemolytic Streptococci		Ratio Volatile to Non-volatile Acid			
Classified according to Holman	Culture	Dex.	Lac.	Sac.	Source
Strep. pyogenes	237 (1)	1: 3.3	1: 4.7	1: 3.9	Erysipelas
" "	237 (2)	1: 3.5	1: 4.5	1: 4.7	"
" "	26 R	1: 6.4		1: 7.1	Mastoid
" "	26 L	1: 3.8	1: 2.4	1: 5.3	"
" "	389	1: 6.4			Hygienic Lab.
" equi	136	1: 4.1	1: 1.4	1: 5.9	Nose
" "	35	1: 2.7	1: 4.0		Wound
" hemolyticus iii	1459	1: 5.7	1: 4.1	1: 6.8	Mulfords' Lab.
" "	388	1: 5.4			Hygienic Lab.
" hemolyticus ii	171	1: 2	1: 5.0	1: 7.0	Antrum
" "	133 a	1: 5.0	1: 3.7	1: 6.1	Throat
" "	133 b	1: 6.3			"
" "	161	1: 6.3			Antrum
" "	1681	1: 7.4			"
" "	169	1: 4.9			"
" "	165	1: 2	1: 3.9	1: 3.2	"
" "	163	1: 2.6		1: 4.7	"
" "	164	1: 5.7		1: 1.9	"
" "	1605	1: 6.4		1: 7.1	"
" anginosus	84	1: 4.6	1: 3.8	1: 6.9	Tonsil
" "	262	1: 4.1		1: 4.1	Empyema
" "	46	1: 3.3			Nose
" "	215	1: 4.7		1: 7.8	Empyema

Table IV continued.

Hemolytic Streptococci Classified according to Holman	Culture	Ratio Volatile to Non-volatile Acid			Source
		Dex.	Lac.	Sac.	
Strep. anginosus	390	1: 5.2			Hygienic Lab.
" subacidus	W-3	1: 6.6			Throat
" "	166	1: 4.5		1: 4.5	Antrum
" infrequens	1613	1: 8.2		1: 4.4	"
" viridans	228 a	1: 6.6	1: 4.3		Endocarditis
" "	228 R	1: 4.2	1: 3.5		"
" "	1313	1: 4.2		1: 10.2	"
" pyogenes	H-1313	1: 5.2	1: 3.2	1: 4.7	Milk
" "	H-23	1: 3.2	1: 4.3	1: 5.9	"

Table V.

Acid Production by Non-hemolytic Streptococci in Dextrose, Lactose, and Saccharose Broths, with the Ratios of Volatile to Non-volatile Acid Content in Each.

Non-hemolytic Streptococci Classified according to Holman	Culture	Ratio Volatile to Non-volatile Acid Dex.	Lac.	Sacc.	Source
Strep. fecalis	I-4	1: 6.2	1: 7.5	1: 8.1	Milk
" "	I-14	1:11.4			"
" "	D-7	1:9.5	1 : 7.8	1: 9.3	"
" "	D-17	1: 6.5			"
" "	D-8	1: 11.4	1 : 11.9	1: 4.9	"
" "	D-18	1: 4.2			"
" "	H-4	1: 3.5	1 : 6.6	1: 10.5	"
" "	I-1	1 : 7.4			"
" "	I-11	1: 1.7			"
" "	I-3	1: 10.1	1 : 3.3		"
" "	I-31	1: 11.5			"
" "	D	1: 10.5	1 : 7.5	1: 8.4	"
" "	J-2	1: 6.6		1: 10.6	"
" "	J-21	1: 9.8			"
" "	J-5	1: 4.5	1 : 4.3	1: 5.3	"
" "	J-15	1: 7.4			"
" "	H-3	1: 8.1		1: 10.5	"
" mitis	J-6	1: 3.8		1: 3.5	"
" "	J-7	1: 7.8	1 : 5.5	1: 7.5	"
" "	J-8	1: 7.1	1 : 3.7	1: 3.3	"
" "	E-1	1: 8.8			"
" "	E-11	1: 5.4			"
" "	E-3	1: 6.2	1 : 15.8	1: 6.0	"
" "	E-10	1: 8.1			"

Table V continued.

Non-hemolytic Streptococci Classified according to Holman	Culture	Ratio Volatile to Non-volatile Acid			Source
		Dex.	Lac.	Sacc.	
Strep. non-hemolyticus i	E	1 : 6.2	1 : 2.3	1 : 6.6	Milk
" "	E-0	1 : 6.9			"
" "	H	1 : 7.5			"
" "	H-0	1 : 6.4			"
" Salivarius	H-13	1 : 7.1	1 : 9.1	1 : 8.2	"
" "	K-2	1 : 8.5			"

isms by their total acidities, by their ratios of volatile to non-volatile acid content, by their specific names and sources of infection all failed to show correlation.

V. DAILY ACID FORMATION.

An attempt was made to discover at what stage in the growth of a culture the greatest production of volatile acids occurred, also if the ratio of volatile to non-volatile acids was constant throughout that period.

1. Methods.

Groups of ten duplicate flasks containing 100 cc. one percent sugar infusion broth were each inoculated with 0.5 cc. of a 24 hour infusion broth culture. These were incubated, titrated each day, and adjusted to the original pH of the broth. One of these flasks was removed daily and steam distilled by the method previously described. The amount of volatile acid found in each distillate was recorded. When the point was reached at which the total acidity was constant for two successive days, and the volatile acid content was stable, the experiment was discontinued and the values plotted.

1. Results.

This method proved satisfactory for the non-hemolytic streptococci and Strep. viridans, but when the curves of hemolytic organisms were plotted, individual differences were noted. For a flask removed on the eighth day of growth might have less acid than the cul-

ture which had been distilled on the seventh day. It was noticeable that the variations occurred in the later days of their growth, as Graphs IV and V show. Curve IV was that of a hemolytic streptococcus from milk, and curve V was that from a human throat.

Graph VI is that of Strep. viridans, and Graphs VII and VIII are of non-hemolytic streptococci isolated from milk.

2. Methods.

In order to correct the fluctuations occurring in the GraphsIV and V, another method was attempted.

Flasks containing one liter of one percent sugar infusion broth were inoculated with 5 cc. of a 24 hour infusion broth culture. 100 cc. were removed each day, titrated, and distilled, the remainder in the flask was adjusted to the original pH of the broth. This procedure was carried on until no further acid production was found to occur. This method proved satisfactory, only greater danger of contamination existed, as the larger flasks were more difficult to handle.

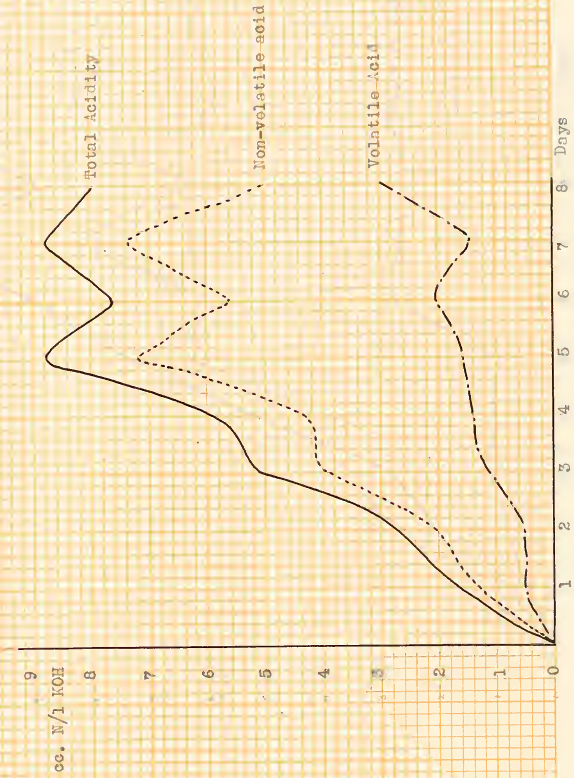
2. Results.

The curves for non-hemolytic streptococci were essentially the same by this method. Graph IX illustrates this point. Graph X of Strep. viridans was fairly similar to Graph VI, although the slope was less in the curves of Graph X than in VI.

The curves XI and XII for hemolytic streptococci show a regular ascent, and are more consistent than

Graph IV.

Daily production of Acid by a Hemolytic Streptococcus from milk - Strep. pyogenes (Holman) No. H-131

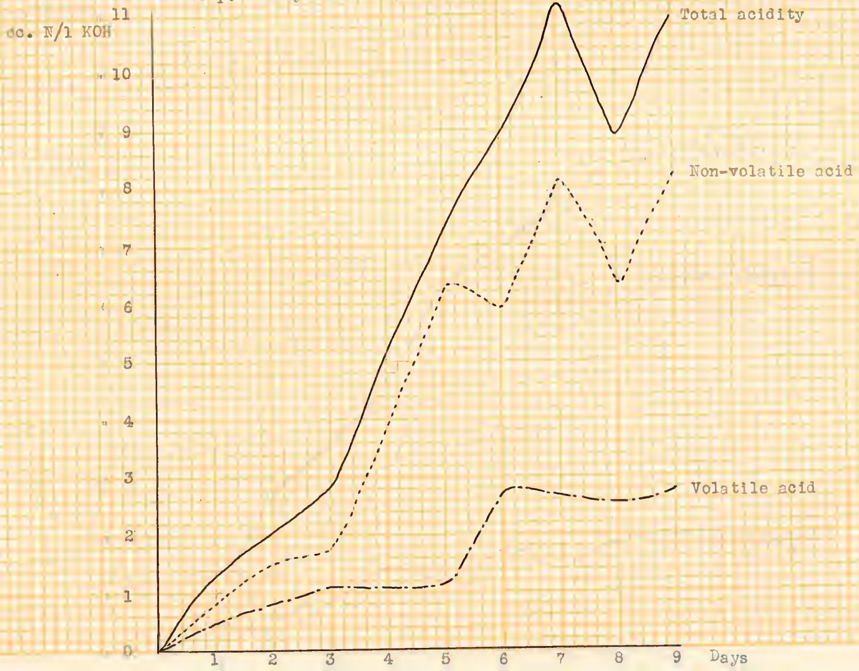


No. 294.

Graph V.

Daily production of acid by a Hemolytic Streptococcus from a throat infection.

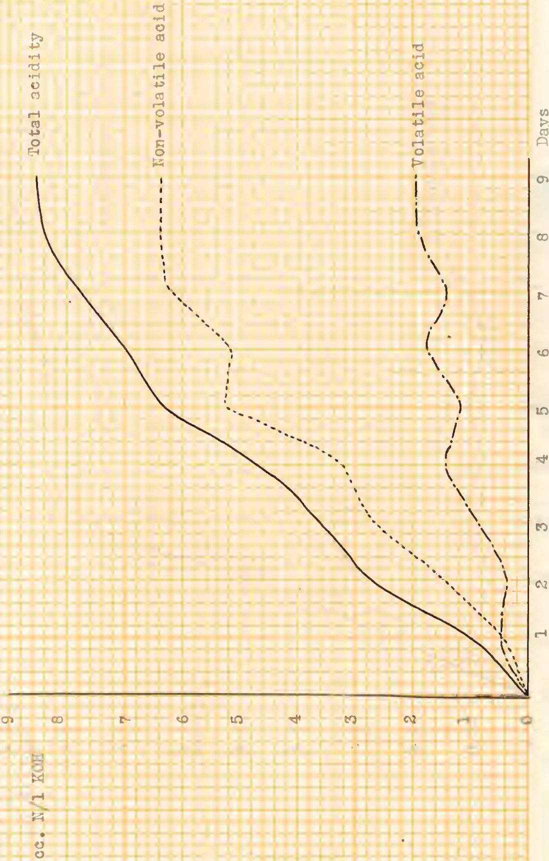
Strep. hemolyticus ii (Holman) No. 133 a.



No. 294

Graph VI.

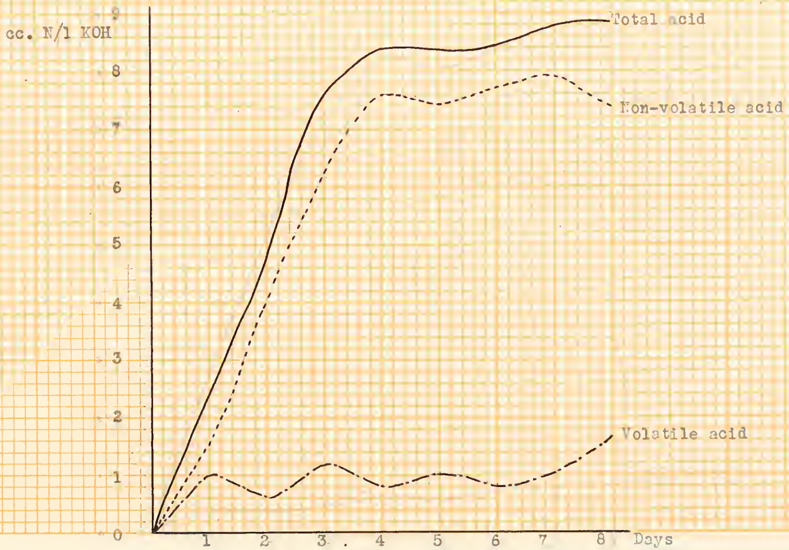
Daily production of acid by a Strep. viridans from a case of Endocarditis. No. 228 a.



No. 294. [illegible]

Graph VII.

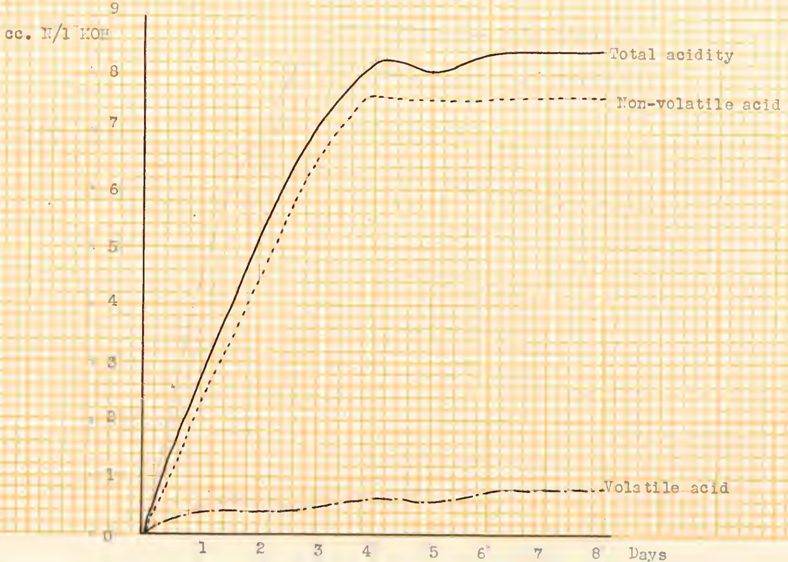
Daily production of acid by a Non-hemolytic Streptococcus isolated from Milk, Strep. mitis (Holman)
No. E-1



Graph VIII.

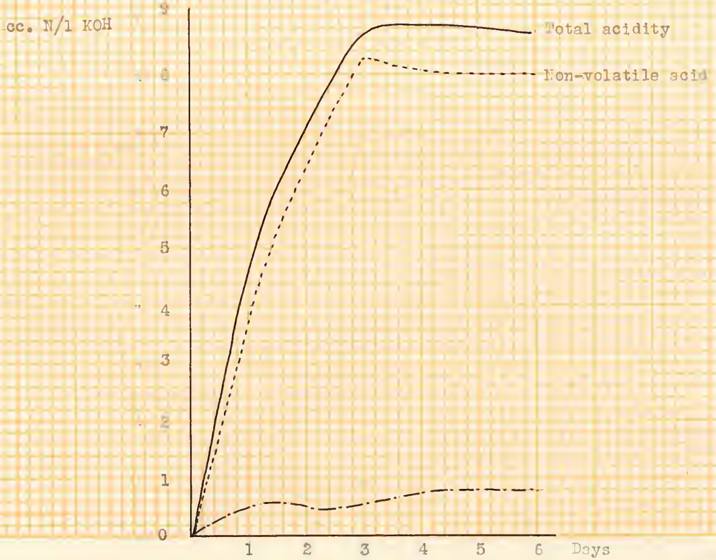
Daily production of acid by a Non-hemolytic Streptococcus isolated from Milk,

Strep. fecalis (Holman) No. D-7.



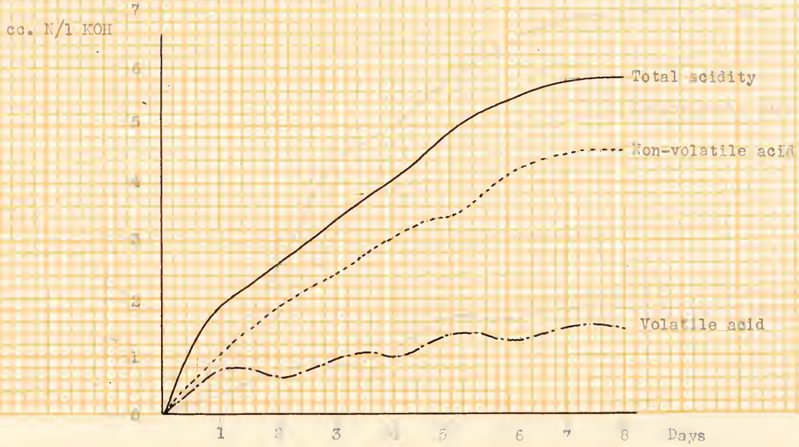
Graph IX.

Daily acid production by a Non-hemolytic Streptococcus isolated from Milk,
Strep. fecalis, (Holman) No. J-5.



Graph X.

Daily acid production by Strep. viridans from a case of Endocarditis, No. 228 R.

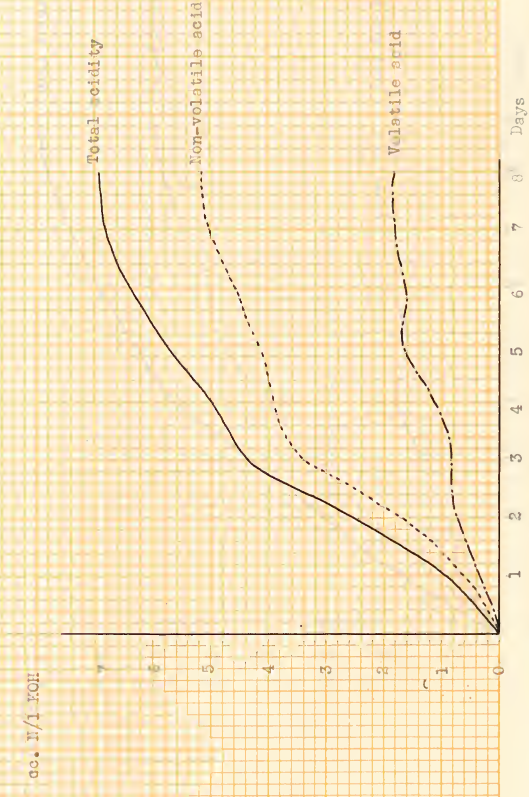


No. 294.

Graph XI.

Acid production of a Hemolytic Streptococcus from an erysipelas infection.

Strep. pyogenes (Holman) No. 37 (1).

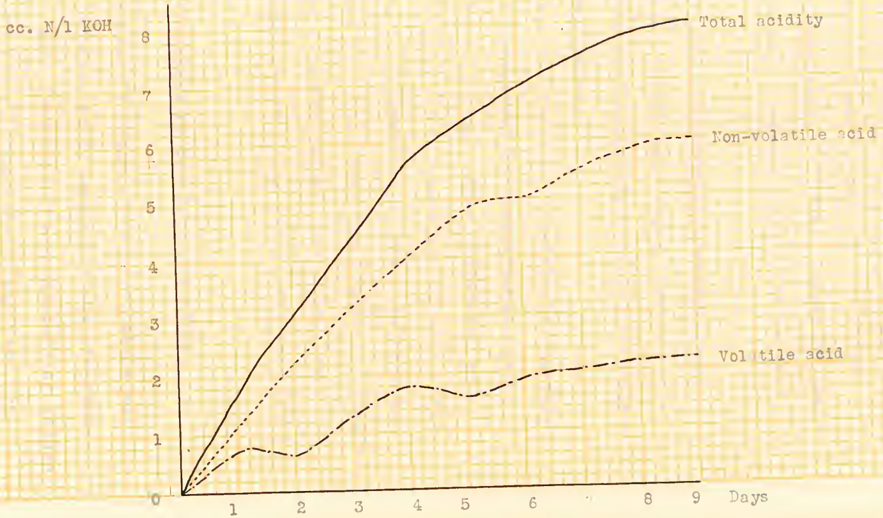


No. 294

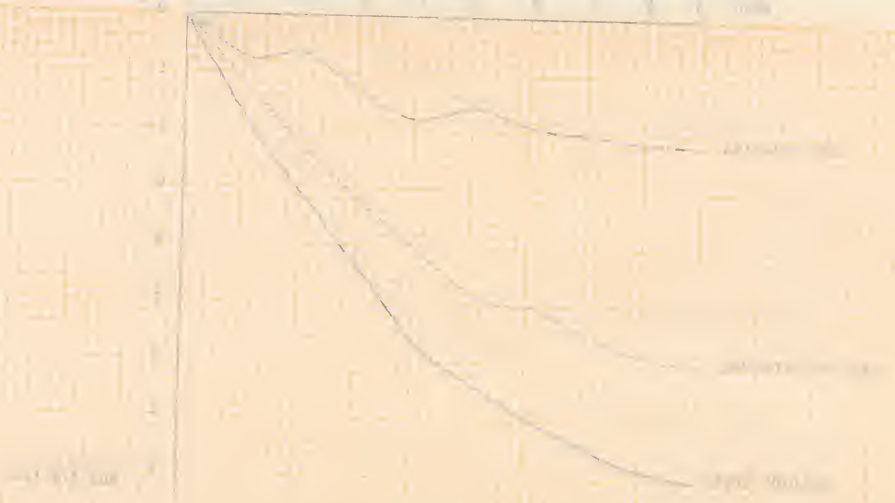
Graph XII.

Daily production of acid by a Hemolytic Streptococcus from an antrum infection,

Strep. hemolyticus ii (Holman) No. 1681.



No 94



[illegible]

[illegible]

[illegible]

Graphs IV and V.

A great similarity was observed throughout the acid development of the individuals in each group. The non-hemolytic streptococci were marked by the rapid rise in total acid production, followed closely by the rise in non-volatile acid content. The volatile acid formation was low.

On the other hand, the hemolytic streptococci did not show as steep a total acid curve, the non-volatile acid did not follow as closely, and the volatile acid production was greater.

In no case was the ratio of volatile to non-volatile acid constant throughout the experiments, as a glance at the graphs will show. Fred, Peterson, and Davenport (1919) stated that in the study of acid fermentation of xylose the proportion of volatile to non-volatile acid proved to be the same throughout the entire ten to twelve days of fermentation; but under the conditions of these experiments, wide variations were found. See Table VI.

VI. RATIOS OF VOLATILE TO NON-VOLATILE ACIDS.

When the respective ratios for the volatile and non-volatile acid production by hemolytic and non-hemolytic streptococci were grouped in their descending values (See Tables VII and VIII) it was found that the non-hemolytic streptococci had a very wide range, while that of the hemolytic streptococci were nearly all con-

Table VI

Ratios of Daily Production of Volatile to Non-volatile Acids

Graph Number	Culture Number	1	2	3	4	5	6	7	8	9 Days
V	H-131	1 to 3	4.7	3.4	3.0	5.7	2.6	5.8	1.5	
VI	133 a	1.6	1.7	1.4	3.4	5.5	1.9	2.8	2.4	2.6
VII	228 a	.6	5.9	2.8	2.1	4.5	2.8	4.3	3.3	3.1
VIII	E-1	1.0	6.8	5.5	10.2	7.0	9.6	8.1	4.3	
IX	D-7	6.6	15.1	15.7	12.6	14.3	9.2	8.0	8.1	
X	J-5	5.9	12.1	17.2	12.3	12.3				
XI	228 R	1.0	3.2	2.3	2.8	2.7	3.3	2.9	2.9	
XII	237 (1)	1.1	2.3	4.3	3.4	2.2	2.4	2.5	2.7	
XIII	1681	1.4	3.3	2.5	2.0	3.1	2.6	2.7	2.7	

fined to the lower ratios, below one to seven, volatile to non-volatile acid production.

a. Ratios arranged in percentage groups.

	Hemolytic Streptococci	Non-hemolytic Streptococci
Dextrose	71% below ratio 1:7	46% below ratio 1:7
Lactose	91% " " "	52% " " "
Saccharose	79% " " "	44% " " "

The averages of all duplicate determinations of total acidities and volatile acid content were taken, and the ratios between the averages found. This smoothed out many of the erratic ratios, for which no explanation could be given, and still showed clearly the great range which the non-hemolytics covered, and the narrow limits of the hemolytic streptococci.

	Hemolytic Streptococci	Non-hemolytic Streptococci
Dextrose	92% below ratio of 1:7	41% below ratio 1:7
Lactose	100% " " "	54% " " "
Saccharose	100% " " "	42% " " "

b. Determination of Mean Ratio.

The ratios obtained from cultures of hemolytic and non-hemolytic streptococci were then submitted to calculation of constants for their frequency distribution. The mean ratio was obtained with its probable error. The standard deviation found was the measure of scatter about the mean. In short, the standard deviation told how much the frequency was spread out on either side of the mean.

Comparison was first made between the two groups of organisms grown on the same kinds of sugar infusion broths. The difference between the mean ratios of hemolytic and non-hemolytic streptococci grown in saccharose broth was 2.053. The deviation which would occur with the existing probable errors, was computed to determine the probability of greater deviations in the difference between the ratios. The probable occurrence of a deviation was only .000023 in each trial. A similar result was found for the difference of 2.296 between the ratios in dextrose broth. In lactose, the difference was greater - 3.472 - but this with its probable error showed a deviation of only .000745 in each trial.

In the case of the hemolytic streptococci, the mean ratio of the acid elaboration in the three sugar broths fell at 4.361± , 5.398± . and 5.743± . The ratio here was not constant, but the average was 5.167± . See Graph XIII.

The mean ratios for the non-hemolytic streptococci ranged between 7.694± and 7.83± , with an average of 7.773± . This showed that irrespective of the carbohydrate fermented, the mean ratio was nearly constant. See Graph XIV. A difference of 2.606 from the average mean ratios of the volatile to non-volatile acid content of cultures in which hemolytic streptococci have been grown, along with the great spread in the standard deviation of the ratios of non-hemolytic streptococci,

Table VII.

Frequency of Ratios of Volatile to Non-volatile Acids produced by Hemolytic Streptococci in Saccharose Infusion broth

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
	1.9	2.7 2.8	3.2 3.6 3.9	4.0 4.1 4.3 4.4 4.5 4.5 4.7	5.0 5.1 5.2 5.3 5.6 5.6 5.7 5.8	6.2 6.2 6.3 6.4 6.5 6.9	7.0 7.1 7.1 7.3 7.8 7.9 7.9	8.6	9.7	10.2					
Distribution	1	2	3	7	8	6	7	1	1	1					Total 37

Frequency of Ratios of Volatile to Non-volatile Acids produced by Hemolytic Streptococci in Dextrose Infusion broth

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
	1.4	2.0 2.0 2.5 2.6 2.6 2.6 2.7 2.8 2.9	3.0 3.1 3.2 3.2 3.3 3.7 3.7 3.8 3.8 3.9 3.9 3.9	4.1 4.1 4.3 4.3 4.3 4.4 4.5 4.6 4.6 4.6 4.7 4.7 4.8 4.8	5.0 5.1 5.2 5.3 5.4 5.4 5.5 5.6 5.7 5.8 5.8	6.1 6.2 6.4 6.4 6.6 6.9	7.0 7.4 7.4 7.4 7.6 7.7	8.1 8.2 8.2 8.6	9.0 9.4 9.5	10.2		12.4	13.0		
Distribution	1	9	12	14	11	6	6	4	3	1		1	1		Total 69

Table VII continued.

Frequency of Ratios of Volatile to Non-volatile acids produced by Hemolytic Streptococci in Lactose Infusion broth

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
	.16 .99	1.4 1.9	2.4 2.6 2.7 2.8 2.8 2.9	3.0 3.1 3.3 3.3 3.4 3.5 3.6 3.7 3.7	4.0 4.1 4.5 4.6 4.7 4.7 4.9	5.1 5.4 5.6 5.9	6.1 6.5 6.7			9.3	10.5			13.4		
Dist.	2	2	6	9	7	4	3			1	1			1		Total 36

FOLD OUT

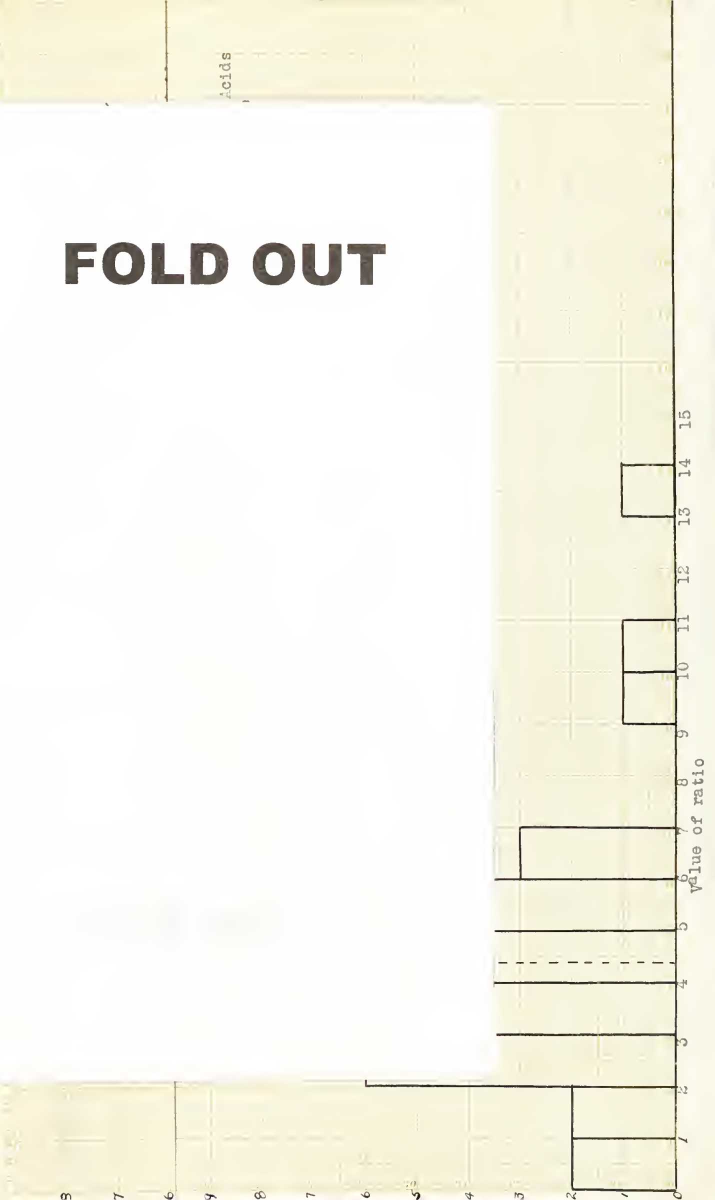

Acids
Value of ratio
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0
1
2
3
4
5
6
7
8
9
6
7
8

Table VIII.

Frequency of Ratios of Volatile to Non-volatile Acids produced by Non-hemolytic Streptococci in Saccharose Infusion broth.

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	1.9	2.1	3.0 3.5	4.0 4.1 4.2 4.3 4.9	5.1 5.1 5.1 5.2 5.5 5.6 5.7 5.8 5.9	6.0 6.2 6.2 6.5 6.7 6.8	7.3 7.8 7.9	8.2 8.4 8.5 8.6 8.6 8.6 8.7 8.9	9.1 9.1 9.1 9.3 9.4 9.6 9.7	10.1 10.2 10.2 10.6 10.7	11.4 11.7	12.2 12.5	13.6 13.6	14.8			
Distribution	1	1	2	5	9	6	3	8	7	5	2	2	2	1	Total 54		

Frequency of Ratios of Volatile to Non-volatile Acids produced by Non-hemolytic Streptococci in Dextrose Infusion broth.

	1.1 1.1	2.0 2.6 2.7 2.7 2.8 2.9	3.2 3.2 3.3 3.7 3.9	4.0 4.1 4.1 4.1 4.2 4.6 4.6 4.6 4.7	5.0 5.0 5.0 5.2 5.3 5.5 5.6 5.7 5.7 5.7 5.8 5.9	6.2 6.2 6.3 6.4 6.4 6.5 6.5 6.5 6.5 6.6 6.7 6.8 6.9 6.9	7.1 7.2 7.2 7.3 7.3 7.4 7.6 7.6 7.6 7.6 7.7 7.7 7.9 7.9	8.1 8.2 8.2 8.2 8.2 8.3 8.4 8.5 8.7 8.7 8.7 8.7 8.8 8.9	9.0 9.1 9.2 9.2 9.4 9.5	10.1 10.4 10.8	11.1 11.2 11.3 11.5 11.7	12.1 12.2 12.3 12.7 12.9	13.8	14.6	15.2 15.4 15.6	16.5	17.1
Distribution	2	6	5	9	12	14	14	14	6	3	5	5	1	1	3	1	1
														Total 104			

Table VIII continued.

Frequency of Ratio of Volatile to Non-volatile Acids Produced by Non-hemolytic Streptococci in Lactose Infusion Broth.

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		19		26
	1.4	2.1 2.5 2.7	3.8 3.8 3.3 3.1	4.5 4.7 4.7	5.0 5.1 5.4 5.6	6.1 6.2 6.4 6.6	7.1 7.9 7.9	8.0 8.1 8.3	9.2 9.3 9.3 9.7		11.6			14.5 14.6	15.7		19.4		26.9
Distribution	1	3	4	3	4	4	3	3	4	0	1	0	0	2	1		1 Total 36		1

FOLD OUT

46
Value of ratio
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21----25
26
1
2

mark a very real difference in the behavior of the two great groups of organisms.

These mean ratios showed that more volatile acid was produced by the hemolytic streptococci in proportion to the non-volatile formed; whereas the non-hemolytic streptococci produced less volatile and more non-volatile acid.

VII. CHARACTER OF ACIDS PRODUCED BY STREPTOCOCCI.

a. Volatile Acids.

In 1921, Foster (1921 c) working with a culture of Streptococcus hemolyticus showed that lactic acid appeared to be the principal acid formed in its fermentation of glucose broth. A smaller proportion of volatile acid is formed. This fraction is made up chiefly of acetic, with perhaps a trace of formic acid.

Evans (1914 and 1918) made a study of the volatile acids produced by two cheese streptococci and Streptococcus lacticus. Some of her data indicated traces of acids of lower molecular weight than acetic - probably formic - and also traces of acids higher than acetic, possibly butyric, which might arise from a slight hydrolysis of the fat in the milk. It seemed justifiable to conclude that acetic acid was the only volatile acid produced by either Strep. lacticus or the cheese streptococci in their normal process of

metabolism in milk culture.

Like Foster (1921) the color tests suggested by Dyer were not found to be successful or reliable, and so were abandoned. Various qualitative tests were applied to the distillate in an attempt to determine the volatile acids present. The following method was resorted to with the hope that it might prove of value.

1. Microscopic Examination of Crystals.

Method: The calcium salts were made by evaporating the neutralized distillate to a volume of 25 cc., acidifying with a few drops of sulphuric acid, warming to liberate any carbon dioxide present, neutralizing with ammonia, and adding a solution of calcium chloride. This was heated to boiling, and a drop put on a slide under the microscope to compare the form of the crystals with those of the known calcium salts of the lower volatile fatty acids. This method was fairly successful but the formation of the crystals had to be carefully watched as they developed, for many forms were observed in their progressive stages.

Results: By microscopic examination of the calcium salts, calcium acetate was found to predominate, and occassionally calcium butyrate crystals appeared. These tests were not felt to be thoroughly reliable, so the volatile esters of the distillate were made.

2. Preparation of Volatile Ester.

Methods: The volatile esters of the acids were made by following the directions of Mullikan (1918).

The neutralized distillate was evaporated to dryness on a water bath. About half a gram of the salt was put in a test tube, 0.1 cc. concentrated sulphuric acid added, and the tube warmed over a small flame until the odor of the vapors of the liberated organic acids could be recognized at the mouth of the tube. After noting whether the odor was simply sharp like acetic or propionic, or sharp and rancid like butyric acid, the tube was cooled, and 0.2 cc. strong ethyl alcohol was added, and then warmed until the vapors again began to come off freely. The tube was then poured into a watch glass containing 5 cc cold water. The odor was much more easily observed in the open watch glass than in a test tube, and the dilution with water removed the sharp smell of sulphuric acid or alcohol which might otherwise mask the more delicate odor of the ester. This was sensibly different for the different acids, but the differences were not great, and the odors may all be described as etherial or fruity. That given by acetic, and resembled closely by the ester from propionic acid, is often spoken of as "refreshing and agreeable". The test is rather delicate, and if made comparatively may suggest which of the four acids were present. More than this could not be expected.

Results: The volatile esters of the acids strong-indicated the presence of both acetic and propionic acids with a slight suggestion of ethyl butyrate in some cult-

ures.

Since the preceding determinations did not include an indicatory test for formic acid, a special procedure was carried out for that unsaturated acid.

3. Test for Unsaturated Acids.

Method: A test for unsaturated acids was applied to a 10 cc. portion of the distillate which had previously not been titrated. A few crystals of sodium carbonate were added to the solution until it was alkaline. A one percent solution of potassium permanganate was added drop by drop. If the purple color of the permanganate was instantly destroyed, formic acid was present.

Results: A reduction of potassium permanganate was obtained in every case where the distillate of a non-hemolytic streptococcus was tested. Negative results were always obtained from the distillates of hemolytic streptococci and Strep. viridans.

Although the last two tests had proved successful, it seemed advisable to make a graphical representation of the volatile acids which were fractionally distilled, and compare the curves of the unknown mixtures with the graphs of known acids.

4. Graphical Representation of Fractional Distillation.

Method: The cultures were steam distilled by the method previously described. The distillate was collected in 10 cc. fractions until 100 cc. had passed over, after which 100 cc. portions were taken off, until 600cc. of the distillate were secured. The distillation was

continued taking only 500 cc. portions, until all volatile acid was entirely removed from the culture. These fractions were then titrated with N/20 KOH, using phenolphthalein as indicator, and the percentage of acid was calculated.

The number of cc. N/20 KOH required to neutralize each fraction of the distillate was divided by the number of cc. N/20 KOH required for the total acid, the result multiplied by 100, giving the percentage amount of acid distilling over into each 100 cc. fraction. Expressed algebraically:

amount of distillate $= x$

amount of acid $= y$

percent acid coming over $= y/x \times 100$

percent acid not over $= (x - y)/x \times 100$

$\log (x - y)/x = (K)\ x$

$\log (1 - (x - y)/x) = Kx$

The last logarithmic expression was the one found to be used by Dyer (1916) to procure the straight line graphic representation of the distilling constants for pure acids. The amount of acid in a given fraction, the "distilling constant" for the fraction, was plotted against the corresponding portion of distillate on arith-logarithmic paper. Pure volatile acids were graphically represented as straight lines and arranged themselves consecutively from the lower to the higher members of the series. With a mixture of two volatile acids, the first

part of the curve occupied a position intermediate to the lines which represented the distilling rates of the higher and lower boiling acids. As the more volatile acid was removed, the curve would gradually become parallel with the line which represented the acid of a lower boiling point.

By plotting a curve from the distilling constants of the distillate of an unknown acid mixture, and comparing it with the curves established for known acids, it follows that the individual acids may be approximately determined.

Results: The logarithmic chart, Graph XV shows in solid lines the straight paths of known acids calculated by Dyer (1916) and plotted from his values of constants. The curves plotted from the distilling constants (shown in Table IX) proved to be so closely analogous to the results of other representatives of the larger groups of streptococci, that only one graph, (XV) will be shown.

The curved paths are characteristic for three groups of streptococci, namely the hemolytic, non-hemolytic, and viridans. The hemolytic streptococci isolated from milk followed the characteristic curve of the pathogenic organisms.

It was realized that this method for analysis of acid mixtures had its limitations. As Gillespie and Walters (1917) pointed out, the method was applicable

to analysis of two or three known acids, but not to mixtures of four known acids, as experimental data was not sufficiently accurate in the later case to give reliable results. By equations computed from Dyer's table of constants, reduction by the method of least squares has been suggested by Gillespie and Walters. If assumptions as to kind and number of acids be wrong, results would be obtained for each acid assumed to be present, although this may sometimes be found absurd as negative results for some sonstituents may be found. Such results do not indicate the absence of the acid for which the absurd result was obtained however, but merely that the original assumptiors were wrong in some unknown particular. If two adjoining acids were known, and a third adjacent acid was suspected, it would be safe to assume a third acid. But this procedure would fail if a fourth acid, not adjacent to the third were present in considerable quantities.

It can readily be observed that a single steam distillation unsupported by sense of smell, or other tests, would not be sufficient to determine the number of acids present in an unknown mixture, unless the number were two, and these were adjacent acids, or ones such as formic and butyric for which distinctive qualitative tests are indicative of their presence.

Hickenbottom (1915), in commenting on Dyer's

Method of calculating a mixture of acids, observed that although a consideration of lagarithmic and other constants would give an accurate idea of the composition of the vapor at any given time, it could not be employed with certainty to decide the compostion of a solution distilled. The method suggested by Dyer of taking the point at which the logarithmic curve of the mixture becomes parallel to that of the less volatile component, was open to a very wide error, and could only be approximated.

Results: The broken line curves in the logarithmic chart substantiated the previous qualitative observations. The distillates from cultures of non-hemolytic streptococci - represented by curve J-5 - always gave a strong reaction for formic acid, and the addition of alcohol and sulphuric acid gave a moderately strong odor of ethylacetate. The slow rate of distillation also substantiated the presence of formic acid. See Table IX.

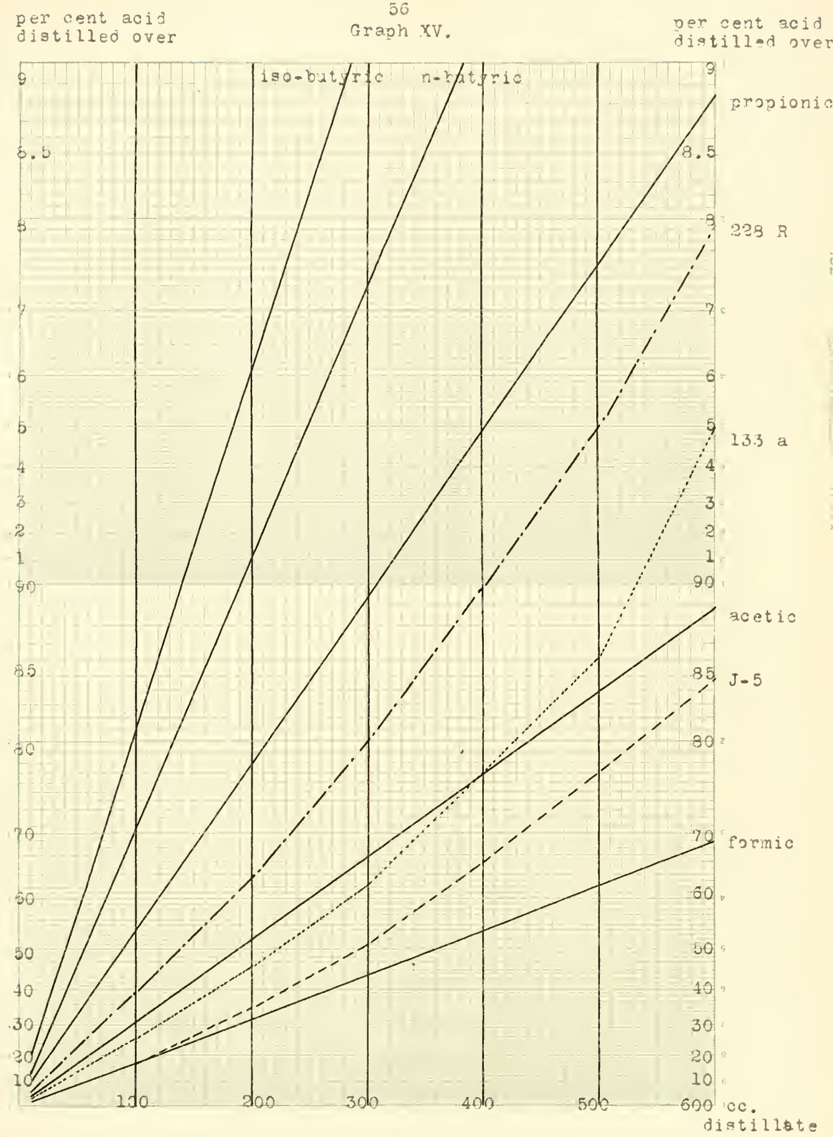
The distillates from cultures of hemolytic streptococci represented by curve 133 a, indicated the presence of formic acid, although all qualitative tests applied to distillates of hemolytic streptococci were negative for that acid. As it was difficult to distinguish between the esters of propionic and acetic acid, it can only be said that either one or the other, or both were present, with a slight additional suggestion of butyric.

Table II.

Distilling Constants of Volatile Acids from Sugar Infusion broth Cultures of Streptococci.

J-[illegible] Non-hemolytic			133 a Hemolytic			228 B. Strep. viridans		
Fraction	cc. N/20 acid	Percent	Fraction	cc. N/20 acid	Percent	Fraction	cc. N/20 acid	Percent
10 cc.	0.9 cc.	1.86 %	10 cc.	1.45 cc.	1.96 %	10 cc.	3.05 cc.	4.74 %
100 cc.	8.60 cc.	17.87 %	100 cc.	19.52 cc.	26.50 %	100 cc.	25.3 cc.	39.36 %
200 cc.	8.50 17.10 cc.	35.54 %	200 cc.	14.37 33.89 cc.	46.00 %	200 cc.	15.51 40.81 cc.	63.50 %
300 cc.	7.70 24.80 cc.	51.54 %	300 cc.	11.79 46.68 cc.	62.00 %	300 cc.	10.61 51.42 cc.	80.00 %
400 cc.	7.00 31.80 cc.	66.09 %	400 cc.	11.42 57.10 cc.	77.49 %	400 cc.	6.43 57.85 cc.	89.95 %
500 cc.	5.55 37.35 cc.	77.63 %	500 cc.	6.30 63.40 cc.	86.04 %	500 cc.	3.21 61.06 cc.	95.00 %
600 cc.	3.55 40.90 cc.	85.00 %	600 cc.	6.60 c 70.00 cc.	95.00 %	600 cc.	1.94 c 63.00 cc.	98.00 %
3,500 cc.	48.11 cc.	100.00 %	2,500 cc.	73.00 cc.	100.00 %	1000 cc.	64.28 cc.	100.00 %

Graph XV.



Logarithmic Chart Showing Straight Path of Individual Acids, and Curved Path of Unknown Mixture.

The cultures of Strep. viridans distilled more rapidly than the cultures of the hemolytic or non-hemolytic streptococci, giving a higher percentage of acid for each fraction. Qualitatively, the distillate was the same as 133 a, but graphically it showed proximity to propionic acid.

b. Non-volatile Acids.

1. Formation of Barium Salts.

Methods: The residue from the steam distillation containing the non-volatile acid, was filtered, evaporated to a volume of about 50 cc., put into a separatory funnel, and shaken with ether many times. The ether extract of the specimens was dark colored and contained some gelatinous matter and fat from the broth. About 50 cc. of water was added to this thick extract, and the mixture heated on an electric plate to drive off the ether. The water solution of the extracted acid was titrated with tenth normal barium hydroxide, adding 5 cc. excess of the base. The solution was boiled fifteen minutes to convert all the lactic acid into its barium salt, after which the excess of barium hydroxide was removed by neutralization with sulphuric acid. This mixture was allowed to stand several hours on the steam bath, filtered to remove the barium sulphate, and the filtrate and washings again shaken in a separatory funnel with ether to remove the elements of broth which still clowded and thickened the solut-

ion. The ether extract was discarded, and the remainder filtered and evaporated to dryness. The residue was dissolved in 100 cc. of water, filtered to remove traces of insoluble organic matter or carbonates, the filtrate was then made up to 100 cc. and divided into two equal parts for analysis. In several samples one portion was tested for succinic acid by making up to 100 cc. with 95 percent alcohol, but in no case was there any precipitation found, even after standing several days. The alcoholic solution was evaporated to dryness in a weighed porcelain evaporating dish over a steam bath and dryed in a sulphuric acid dessicator until constant weight was secured. An excess of sulphuric acid was added and the salts converted into barium sulphate by ignition. From the weight of barium sulphate the corresponding weight of lactic acid was calculated.

Results: Gravimetric determination of the kind and proportion of non-volatile acid was made of two hemolytic and two non-hemolytic cultures. The results show lactic acid to be probably the only non-volatile acid present.

The differences between the calculated amount of normal lactic acid and the amount of non-volatile acid actually found in the culture by the subtraction of the volatile acid from the total acidity, may be due to the presence of another non-volatile acid in

small amounts, or to experimental error accentuated by the repeated filterings necessary to clear the solution of the elements of broth which constantly clouded it.

Lactic Acid as Determined by Barium Salt Method.

Hemolytic	Weight of Ba Salts	Weight of $BaSO_4$	Calculated Amount Lactic Acid	Actual Amount of Non-volatile acid Titrated in Culture
237L-3	0.98 gr.	0.71 gr.	6.18 cc.	6.64 cc.
133a-3	1.46 gr.	1.06 gr.	9.31 cd.	9.90 cc.
Non-Hemolytic				
J-7-4	1.52 gr.	1.12 gr.	9.77 cc.	10.40 cc.
D 1	1.50 gr.	1.09 gr.	9.50 cc.	9.71 cc.

2. Formation of Zinc Salts with Determination of Rotary Type.

Methods: The zinc salts of lactic acid have been used by a number of investigators to determinate both the quantity and the form of the acid produced. To the second part of the divided filtrate - not used in the barium salt determination above - were added a few drops of sulphuric acid and zinc carbonate equal in grams to one half of the tenth normal barium hydroxide used in titrating the entire sample of non-volatile acid. The solution was boiled for ten minutes to drive off the carbon dioxide. Five cc. were withdrawn, centrifuged, and the

clear supernatant fluid tested with sulphuric acid for unprecipitated barium. The sample was then returned to the beaker, and more sulphuric acid and zinc carbonate added if necessary. After standing over night, the solution was filtered to remove the barium sulphate. The filtrate and washings were evaporated on a steam bath until crystals began to form. The dish was placed in a sulphuric acid desiccator for several days until crystalization was complete. The crystals were transferred to a hardened filter paper, washed with 5 cc. cold water, gentle suction being applied. An additional crop of crystals was obtained from the mother liquor and washings. The crystals were dried for several days in a desiccator, then removed to a weighing bottle and dried to constant weight over sulphuric acid.

Fred and Peterson (1919) state that in the spontaneous fermentation of sugar solution the usual form of lactic acid produced is the racemic type. One means of determining the form of acid is by finding the amount of water of crystallization contained in the zinc salt. The racemic zinc lactate crystallizes with three molecules of water, constituting 18.7 percent of the weight of the salt, and the active zinc lactate with two molecules, forming 12.9 percent of the weight. By heating for several hours in the electric oven at 105° - 108° this water of crystallization was driven off. By difference in weight before and after heating

the percentage loss of water calculated.

Norris (1912) states that the d-lactic acid is a characteristic constituent of muscle, but when heated it loses its optical activity and racemic acid is formed; l-lactic acid is obtained by the fermentation of sugar - this latter statement disagreeing with the statement of Fred and Peterson (1919). Plimmer (1918) claimed that lactic acid may be either dextro-rotary or laevo-rotary, or inactive, according to its source; certain bacteria produce laevorotary lactic acid.

Owing to the fact that the lactic acid is contained in the residue left over from the distillate of the volatile acid, and has been boiled many hours, the racemic form would be expected.

Results: Two determinations were made to find the percentage of water of crystallization in the zinc salt. 17.2 percent and 17.7 percent of the weight of the samples were water, showing that it was the racemic zinc lactate which crystallizes with three molecules of water and theoretically constitues 18.7 percent of the weight of the salt.

In the light of the ratios found, the qualitative, quantitative, and graphical experiments performed in the determination of the fermentation products, it may be said that the hemolytic streptococci produced more acetic, propionic, and possible butyric acids in proportion to the lactic acid found. The non-hemo-

lytic are the reverse, for they produce more lactic in proportion to the formic, acetic, and likely butyric acids present.

VIII. CONCLUSIONS.

1. During the first 24 hours of growth in a saccharose, dextrose, or lactose infusion broth, the acid production by non-hemolytic streptococci was found to be nearly twice as great as the acid production by hemolytic streptococci.

2. Titratable acidity and hydrogen-ion content are only comparable in cultures grown in the same lot of broth.

3. Titratable acidity and hydrogen-ion content fall within definite limits, and are equally satisfactory in differentiating the fermentation of hemolytic and non-hemolytic streptococci.

4. Total acidity at the end of the acid production period in 100 cc. one percent sugar infusion broth, was about equal in the two groups of streptococci.

5. The hemolytic streptococci required a longer period of time in which to bring about a complete conversion to acid of the one percent sugar of the broth.

6. All hemolytic streptococci showed a gradual rise in daily total acid production with a low ratio of volatile to non-volatile acid content.

7. All non-hemolytic streptococci were alike in showing a rapid rise in total acidity during the first days of fermentation, with a low production of volatile acid, giving a high ratio of volatile to non-volatile acid content.

8. The ratio of volatile to non-volatile acid was determined for 30 non-hemolytic and 32 hemolytic streptococci. The mean ratio for the hemolytic streptococci was 1 to 5.167 ± with a comparatively small deviation. The mean ratio for the non-hemolytic streptococci was 1 to 7.773 ± with a wide deviation.

9. The non-volatile acid produced was almost entirely lactic acid of the racemic type.

10. Non-hemolytic streptococci produced formic, acetic, and a possible trace of butyric acid.

11. Hemolytic streptococci and Strep. viridans produced acetic and propionic acids. Butyric acid was probably present in small quantities. All tests for formic acid were negative.

12. The acids produced by hemolytic streptococci isolated from milk were like those formed by the hemolytic streptococci which had come from pathogenic sources.

13. Streptococci isolated from similar pathogenic lesions, or bearing like specific names, showed no constancy of ratio between the volatile and non-volatile acids which they produced.

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BIOGRAPHICAL SKETCH

Bertha Langwill was born in Owen Township near Rockford, Illinois, October third, 1895. After graduating from the common schools, she attended Rockford College for Women, from which she received a Bachelor of Science Degree in June 1916, with majors in Biology and Chemistry. During the following year she taught in the public schools of Rockford, Illinois. The summers of 1915 and 1916, and the year 1917-18 were spent at the University of Illinois; in June 1918 she was granted the Master of Science Degree from that university, with Zoology as major. During the next two years she taught Chemistry and Biology at Sweet Briar College, Sweet Briar, Virginia. The summer of 1919 was spent at Puget Sound Biological Station, Friday Harbor, Washington, and the following summer she taught mathematics in the summer school at Rockford, Illinois. During the year 1920 1921 she taught Biology at Elmira College, Elmira, New York, and in October 1921 entered the School of Hygiene and Public Health of Johns Hopkins University as a candidate for the Doctor of Science Degree with Bacteriology as major.